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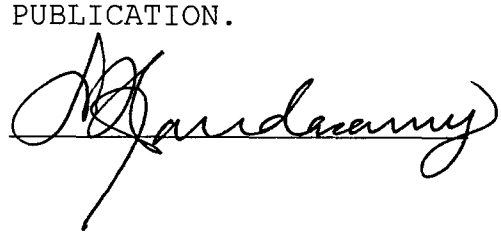

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13. ABSTRACT ***(Maximum 200 Words)***

In order to better understand the role of estrogen metabolism as it relates to breast cancer etiology, a new analytical technique that can measure CE and CE-DNA adducts at low endogenous levels is being developed. This new technique is based on HPLC analysis of fluorescent probes specific for CE and CE-DNA adducts. Reaction of α,α-dibromomalonates occurs quickly with catechols, and this malonate system is being developed to produce fluorescent probes for HPLC analysis. Synthesis of bis-(9-methylenefluorenyl) α,α-dibromomalonate and its reaction with catechol and 4-OHE_1 produced fluorescent products whose emission was red-shifted from 315 to 455 nm. This red-shift was also accompanied by reduced quantum yield that limits the sensitivity of the fluorenyl malonate probe. This derivation was also analyzed by fluorescence line narrowing spectroscopy (FLNS) to see if structural differences could be observed. FLNS showed that small changes in A ring environments of catechol and 4-OHE_1 could be detected. In order to increase the sensitivity of malonate derived CE, bis-(9- methyleneanthranyl) α,α-dibromomalonate was synthesized. Reaction of the anthranyl malonate probe with CE is ongoing. The long-term goal for developing this new analytical assay is the establishment of biomarkers for the early detection of breast cancer.

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INTRODUCTION:

The metabolism of estrogen to procarcinogenic catechols has been hypothesized as an initiation step in the development of breast cancer [1-4]. Specifically, the over expression of 4-hydroxylase activity has been observed in organs prone to estrogen-induced tumors [5-8]. The role of 4-hydroxyestradiol (4-OHE_2) and 4-hydroxyestrone (4-OHE_1) metabolites with the increase occurrence of estrogen-induced tumors is still not clear. Hypothesis regarding redox-cycling [9-13] and oxidation to electrophilic quinones have been examined [3,4]. Since oxidation of catechol estrogens (CE) to catechol estrogen quinones (CE-Q) has been shown to lead to CE- DNA adducts [4], we seek to develop an analytical technique that can measure these adducts at biologically meaningful, endogenous levels. The purpose of developing this assay is to examine whether CE-DNA adducts are present in breast cancer tissue. This would be a first step in understanding the etiology of breast cancer as it relates to estrogen metabolism; specifically, the role of 4-hydroxylase activity in the increase occurrence of estrogen-induced cancers. The scope of this method development involves the production of fluorescent probes, specific for the catechol moiety, so that ultra, low-level detection of these adducts can be accomplished. With the proper development of fluorescent probes, this assay would not only be very sensitive, but selective towards the oxidatively liable CE-DNA adducts.

BODY:

Our work the past year was focused on accomplishing Task 2, "Develop an HPLC analytical procedure, via pre-column fluorescence derivation, for the femtamolar detection of CE-adducts, CE, and MPEM in human breast tissue". The production of novel *gem*-dihalides, reactive towards the catechol moiety, was advanced in order to find a fluorescence probe that would not only allow for sufficient sensitivity, but also help to identify estrogen metabolites. Since the completion of Tasks 1 and 3 depends on this analytical probe, we have spent our efforts engineering a probe that will significantly advance the technology of detecting CE metabolites and CE-DNA adducts in human tissue.

The production of a new fluorescent probe specific for the catechol moiety requires the consideration of several issues. Quantum yields for fluorescent molecules are highest in ridged systems with minimal conformational flexibility near the fluorescent emitting portion of the molecule [9]. The binding of both phenol hydroxy groups onto a single atom generates a new ring system with reduced conformational flexibility when compared to the binding of two separate fluorescent probes (Figure 1). Thus, the design of our new probe was conducted with several features in mind. First, a highly fluorescent fluorophore was selected to maximize sensitivity. Second, the fluorophore should be attached to an atom baring two electrophilic leaving groups so that reaction with both nucleophilic phenol hydroxy groups could take place leading to the formation of a five membered ring. Third, the atom possessing the two electrophilic leaving groups could not be prochiral because formation of a new chiral center would produce a mixture of diastereomeric products (since the estrogen ring system is chiral), complicating separation and quantitation of CE-DNA adducts. Fourth, th reaction with phenols should take place in near quantitative fashion, under mild conditions, in solvents conducive to reverse phase HPLC. Also, if the probe is design in a manner that allows interaction between the CE A ring and the fluorphore, fluorescence line narrowing spectroscopy (FLNS) can be used to identify the metabolite based on standard spectra.

Reaction of catechols with *gem*-dihalides has been our approach to satisfying the above criteria. Reaction of 9,9-dibromofluorene (DBF) with catechol leads to a spiro fused ring system that contains a ketal functional group (Figure 2). This reaction proceeds in good in the presence of anion exchange resins to produce the ketal product. Unfortunately, the ketal product displayed no measurable fluorescence. Interaction of lone pair electrons from the ketal oxygens with the fluorene ring system may be responsible for the fluorescence quenching.

The facile reaction of catechol with DBF led us to try other activated, fluorescent *gem*-dihalides for catechol derivation. Methylene groups located between two carbonyls are easily brominated to produce *gem*-dibromides. Investigations into the reactivity of these *gem*-dibromides with catechol were

done using ethyl α,α–dibromomalonate as a probe. Exploring several different combinations of base and solvent, it was found that reaction of α,α–dibromomalonate with catechol in the presence of Cs_2CO_3 (or CsF) in *N,N*-dimethylforamide (DMF) led to high yields of the ketal product (Figure 3). The reaction is complete after 20 minutes at room temperature. Since various esters of the malonate core could be synthesized from malonic acid, we chose this *gem*-dihalide system to build an appropriate fluorescence probe for CE.

The commercially available 9-fluorenylmethanol was mixed with malonic acid in toluene in the presence of *p*-toulenesulfonic acid. Dean-Stark removal of water over 24 h led to the difluorenyl ester, bis-(9-methylenefluorenyl) malonate (Scheme 1). This ester was bromonated in chloroform using pyridinuim bromide perbromide and triethylamine to produce bis-(9-methylenefluorenyl) α,α–dibromomalonate (Scheme 1). Reaction of bis-(9-methylenefluornyl) α,α–dibromomalonate with catechol, using the reaction conditions presented above, led to a ketal product that displayed fluorescence not at the usual 315 nm emission of fluorene, but instead displayed an red-shifted emission at 455 nm (Figure 4). This longer wavelength emission also displayed a lower quantum yield than the fluorene parent compound indicating quenching of the fluorophore had occurred.

Investigation into the fluorescent behavior of catechols derived from bis-(9-methylenefluorenyl) α,α–dibromomalonate was done by submitting the compounds to low temperature FLNS. Both catechol and 4-OHE_1 were derivatized with bis-(9-methylenefluorenyl) α,α–dibromomalonate, the products separated by HPLC, and the corresponding HPLC fractions sent to Rsyzard Jankowiak at Iowa State University for FLNS analysis. Fluorescence spectra of the two derivatives at 77K shows that their emission spectra differ in the 450 nm region (Figure 5). This indicates that malonate fluorophores could be developed such that FLNS could distinguish differing environments of the catechol A ring. CE metabolites and CE-DNA adducts contain significant differences in substitution at the catechol A ring. Thus, these malonate probes should be useful for structure identification based on authentic standards.

The reduced fluorescence emission from the bis-(9-methyleneflourneyl) α,α–dibromomalonate derived catechols could be the result of fluorescence resonance energy transfer (FRET). FRET occurs when the emission frequency of one molecule overlaps with the absorption frequency of another, near-by molecule [10]. The emission spectrum of fluorene is centered around 315 nm, this is close to the adsorption maximum of 280 nm for catechol. The resulting emission of the derived products at 455 nm indicates interaction between the catechol ring and fluorene. Bis-(9-methyleneflourneyl) malonate, the precursor ester that contains no catechol structure at the α carbon, displays fluorescent properties very similar to fluorene. The incorporation of a catechol ring, with catechol or 4-OHE_1 derivation, leads to a red-shift in the emission to 455nm perhaps as the result of extended conjugation between the two fluorene rings through the catechol ring. Other factors such as π–π interactions have not been ruled out.

In order to test the hypothesis the reduced quantum yields and red-shift of the emission is due to a FRET phenomenon, we plan to change the fluorophore on the malonate ester core to longer wavelength emitters. Anthracene, coumairn, and pyrene fluorophores all have emission spectra close to 400 nm, well separated from the 280 nm absorption of catechol. We have synthesized bis-(9-methyleneanthranthranyl) α,α–dibromomalonate. The synthesis of the anthracene probe required a new synthetic methods since bromination of the bis-(9-methyleneanthranyl) malonate ester resulted in an unknown product probably due to bromination of the anthracene ring system (Scheme 2). Thus, bromination of malonic acid, followed by ester formation with 9-(chloromethylene)anthracene produced bis-(9-methyleneanthranyl) α,α–dibromomalonate in good yield. We are currently reacting this probe with catechol and CE to establish the fluorescence properties of the derivatized products. Using other known probes for carboxylic acids, we are modifying α,α-dibromomalonic acid with coumairn and pyrene ring systems. These ester probes will use to form CE derivatives and their spectra will be analyzed using FLNS.

KEY RESEARCH ACCOMPLISHMENTS:

- Establishment of reaction conditions needed to couple catechols with α,α-dibromomalonate esters.
- Synthesis of bis-(9-methylenefluornyl) α,α–dibromomalonate and derivation of catechol and 4-OHE_1 with this new fluorescent probe.
- Synthesis of bis-(9-methyleneanthranyl) α,α–dibromomalonate.

REPORTABLE OUTCOMES:

1) Poster presentation, "Fluorescent Probes for Catechol Estrogens Using Esters Of α,α-Dibromomalonate", Era of Hope Meeting, Atlanta Georgia, June 9, 2000.
2) Two Undergraduates, Clark Diffendaffer and Nicole Burns were employed full time during the summer of 1999 and during the academic year 1999/2000. Their training in synthetic processes and analytical procedures not only provided for summer and part-time academic year employment, but also furthered their academic goals relating to careers in health care and cancer research.

CONCLUSIONS:

In order to produce an analytical assay capable of measuring endogenous levels of CE-DNA adducts, fluorescent probes specific for CE estrogens are needed. A successful design for these fluorescent probes would allow for CE-DNA detection at the femtamolar level. This requires the probe be selective for the catechol ring system. Since the catechol ring system contains vicinal, weakly nucleophilic oxygens, probes based on reactive gem-dihalides would allow for selective coupling of the catechol ring to the probe. Thus, design of fluorescent probes containing reactive gem-dihalides has been a central focus of our research.

Reaction of 9,9-dibromofluorene with catechol occurs in high yield, at room temperature, in DMF to produce a ketal product. The resulting spiro ring system displayed no measurable fluorescence. It was discovered that α,α-dibromomalonates also react with catechol in high yield in DMF in the presence of Cs_2CO_3. The incorporation of various fluorescent groups into the malonate core was explored to produce fluorescent probes for catechol and CE.

Bis-(9-methylenefluorenyl) α,α-dibromomalonate was synthesized from 9-fluorenylmethanol and malonic acid in two steps. This new malonate probe reacts with catechol to produce a fluorescent product that displays a red-shift emission (455 vs. 315 nm) with respect to the fluorene fluorophore. This longer wavelength emission displays reduced sensitivity when compared to fluorene; however, the interaction of the catechol ring system with the fluorene ring system could allow for product identification via FLNS. FLNS spectra of both catechol and 4-OHE_1 derivatives of bis-(9-methylenefluorenyl) α,α-dibromomalonate displayed spectral differences in the 450 nm region indicating that fluorescent probes based on fluorescent malonate esters could be used to identify CE-DNA adducts using FLNS.

In order to increase the sensitivity of fluorescent malonate esters, longer wavelength emitters were incorporated. Bis-(9-methyleneanthranyl) α,α-dibromomalonate was synthesized from 9-(chloromethylene)anthracene and malonic acid in three steps. The reaction of this probe with catechol and CE is ongoing and will be the focus of future work in the coming year.

The production of a viable analytical assay that can measure DNA damage caused by estrogen metabolites is an important first step in establishing biomarkers for early breast cancer detection. With

the development of this new assay, one can seek to establish a link between the production of CE-DNA adducts and the onset of breast cancer. If this link were established, biomarkers for the early detection of breast cancer would be at hand. In addition, as new analytical tools for measuring various estrogen metabolites develop, the role of CE estrogen metabolism, as it relates to breast cancer etiology, will become more clearly understood. This could lead to strategies aimed at preventing breast cancer.

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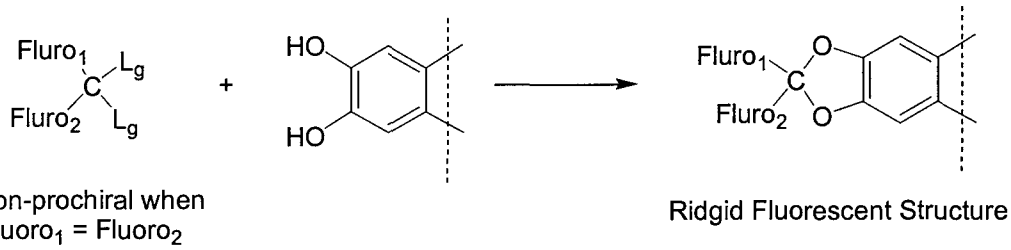
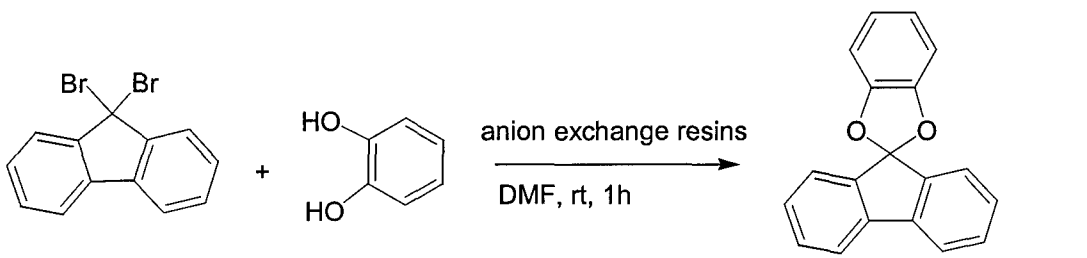
APPENDICES:



non-prochiral when
$Fluoro_1 = Fluoro_2$

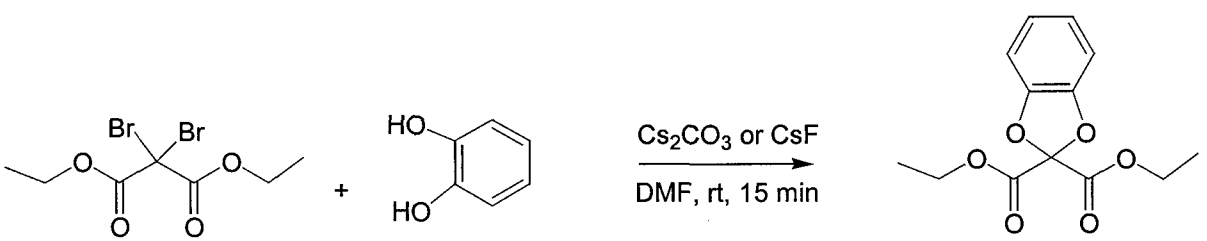
Ridgid Fluorescent Structure

Figure 1. Structural features of a successful fluorescent probe.



non-fluorescent ketal product

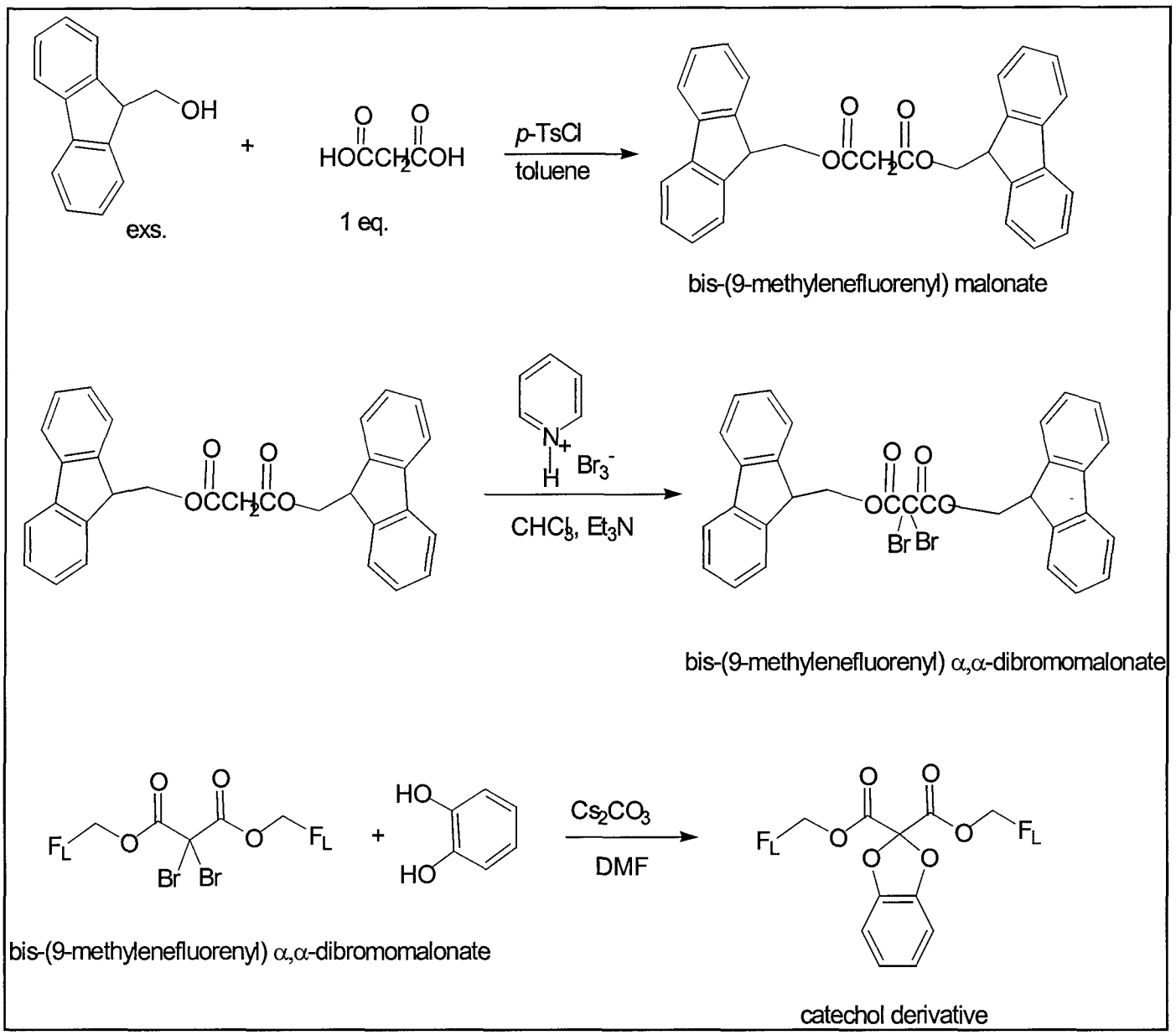
Figure 2. Catechol derivative of 9,9-dibromofluorene.



2 to 4 eq.

> 90 % yield

Figure 3. Catechol derivative of ethyl α,α-dibromomalonate.



Scheme 1: Synthesis and reaction of bis-(9-methylenefluorenyl) α,α-dibromomalonate

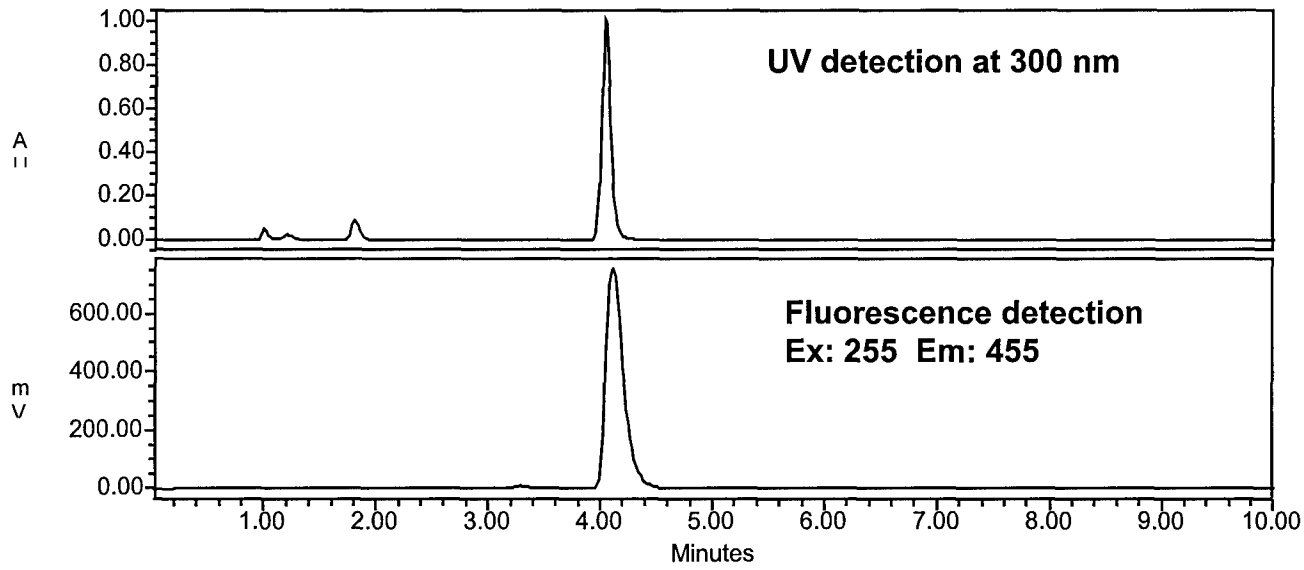


Figure 4. Chromatograph using UV detection (top) and fluorescence detection (bottom) of catechol derived with bis-(9-methylenefluorenyl) α,α-dibromomalonate.

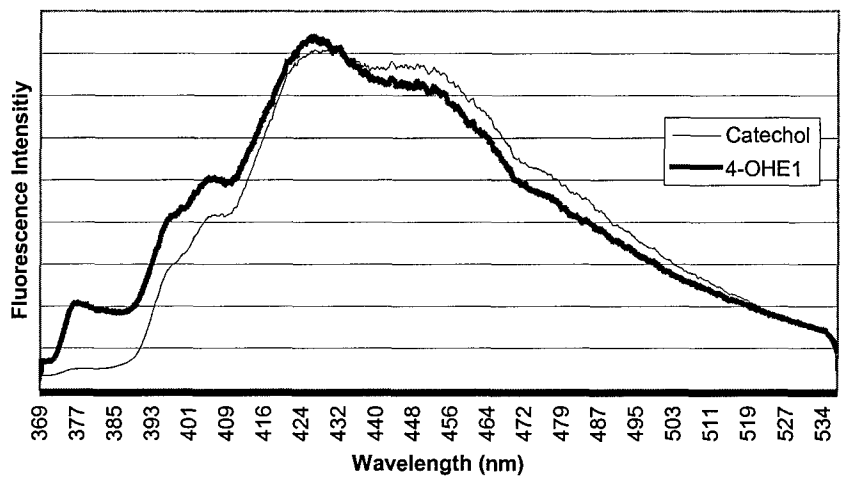
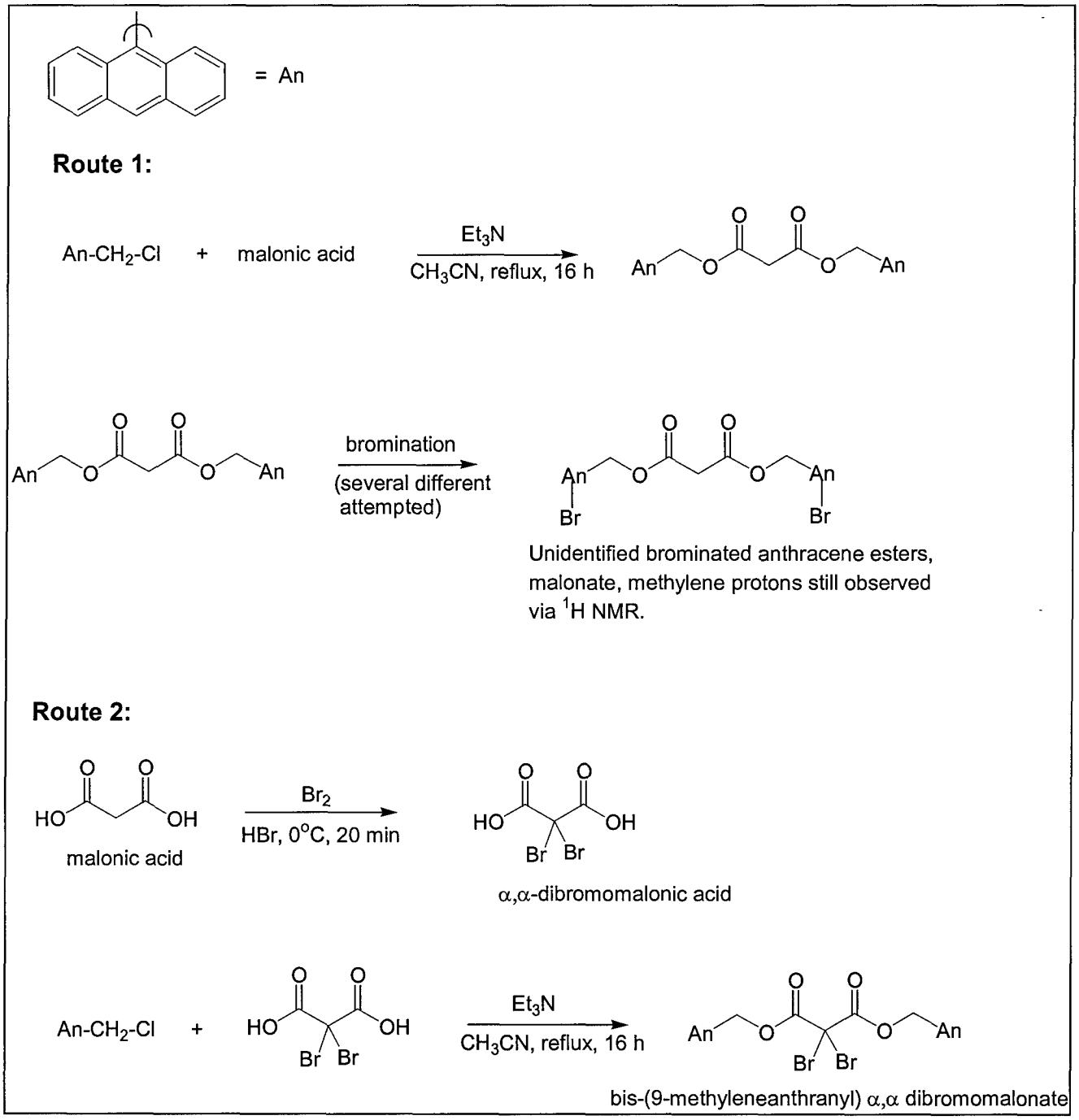


Figure 5. Low-temperature fluorescence spectra (77K) of catechol and 4-OHE_1 derivatives.



Scheme 2. Synthesis of bis-(9-methyleneanthranyl) α,α-dibromomalonate.

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